Laboratory assessment of antithrombotic therapy

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Laboratory Assessment of Antithrombotic Therapy: What Tests and If So Why?

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Abstract

A critical review is given of the tests available for the assessment of the action of anticoagulants, such as heparins, oral anticoagulants and direct thrombin inhibitors, in patients under antithrombotic therapy. The principle of action and the performance of the thromboplastin time (PT), the activated partial thromboplastin time (aPTT), the whole blood clotting time, the thrombin time, the ecarin clotting time and the endogenous thrombin potential (ETP) is discussed, as well as the evidence behind the accepted therapeutic ranges. The two most common tests, PT and aPTT, respond in an essentially different way to clinically effective anticoagulation with heparin and with oral anticoagulants. This means that they covariate with, but do not themselves represent the essential parameter influenced by anticoagulation. The experimental basis for the widely accepted two times prolongation of the aPTT as an indicator for adequate anticoagulation is shown to be meagre in the case of unfractionated heparin and lacking for the other anticoagulants. Common sources for error in the interpretation of anti-factor Xa- and anti-thrombin activity of heparins are indicated. Extensive experience with new tests like the ecarin clotting time and the ETP is still lacking. On the basis of preliminary data and in view of the importance of the enzymatic action of thrombin in the pathogenesis of thrombosis, the ETP is considered a possible candidate for a common parameter to assess different types of anticoagulants.

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Two classes of anticoagulant agents are in current use today, the heparins and the oral anticoagulants. Within the heparin group, we distinguish unfractionated heparin (UFH) that accelerates the inactivation of both thrombin and factor Xa and low-molecular-weight heparins that lack the very high molecular weight fractions (>10,000) and contain significant amounts of a component that only fosters the inactivation of factor Xa. Oral anticoagulants inhibit the synthesis of functionally active carboxylated vitamin-K-dependent coagulation factors (factors II, VII, IX, X, proteins C and S). During the last decade, a third class of substances has been developed: direct specific thrombin inhibitors, such as hirudin, hirulog, argatoban and several others [1].

One of the key points in the clinical development of a new antithrombotic agent is to determine the optimal dosage. The proof of the pudding remains in the eating: i.e. the ultimate test are clinical dose-finding studies that relate the antithrombotic effect to the plasma levels of the anticoagulant. Such studies are extremely costly in time and money and fairly inaccurate in determining the range of concentrations that enables adequate treatment. It is therefore of utmost importance to dispose of a laboratory model that can serve to relate pharmacokinetics to the anticoagulant effect.

There were two fundamentally different types of such models. The animal thrombosis model, in which thrombosis is provoked in any of a number of standardized ways, and the relationship between drug level and thrombosis prevention can be determined, if required to a high degree of accuracy. The problem with this approach is that the data on artificial thrombosis in an animal should be used to guess the dose relationship in clinical thrombosis in man. This article is not meant to discuss this problem further.

The second type of model is an ex vivo test that can be carried out on the blood of a patient and that varies with the level of antithrombotic action obtained on the pathogenetic mechanism in the patient. An ideal test would be that one that directly reflects the effect of the drug on the relevant pathogenetic mechanism. Tests that only covariate with the antithrombotic action are still useful as long as the correlation with the pathogenetic mechanism is high and the tests meet general standards of reproducibility and accuracy. This raises the question of what indeed is the mechanism of action of the anticoagulant drugs. The fact that the three groups of drugs listed above each have an entirely different mode of action, facilitates an educated guess: it will not seem far fetched to surmise that the one thing that they have in common is the essential property that determines their antithrombotic action. That evidently is their capacity to diminish the amount of free thrombin that develops in clotting blood.

The next question, and the main one that we want to discuss in this review is: what are the relative merits of the currently used ex vivo tests for monitoring anticoagulant/antithrombotic treatment [2].

Thromboplastin Time or Prothrombin Time Test (PT)

The principle of the PT is that it measures the time necessary to clot a plasma sample after recalcification in the presence of an excess of tissue factor and procoagulant phospholipid, i.e. the clotting time in the extrinsic system, in which the factors I, II, V, VII and X are involved. Since the introduction of oral anticoagulants in the early 1940s, the PT has been the primary and exclusive means of monitoring the effectiveness and safety of oral anticoagulant therapy. Its results can be ex-

pressed in seconds, in the prolongation ratio (i.e. seconds in the sample/seconds in a normal control) or in percent activity, i.e. the clotting factor concentration in diluted normal plasma that will cause an identical clotting time. The results vary with the type of thromboplastin used, no matter what way of expression is used. Also, as a rule, calibration curves of diluted normal plasma are not parallel to those of diluted samples of anticoagulated blood and are dependent upon the dilution medium used. Hence, it is not possible to determine percent activity unequivocally. Therefore, the ratio method is commonly preferred. Variabilities in the results obtained with different thromboplastin reagents may cause oral anticoagulant therapy to be managed inappropriately [3]. In response to these problems, the World Health Organization (WHO) recommends a mathematical correction of the PT results known as the International Normalized Ratio (INR). The INR relies upon the calibration of commercial thromboplastins against a standard reagent with a known sensitivity to the antithrombotic effect of oral anticoagulants. By reporting PT as INR, results from laboratory to laboratory can be compared with acceptable accuracy. Today, this reporting technique is recommended for the monitoring of oral anticoagulant therapy. Extensive studies have been carried out to express the level of safe and efficient oral anticoagulant therapy in terms of INR [4, 5]. Nevertheless, a number of potential problems with the INR were identified. On the one hand, even the most extensive studies leave large margins of incertainty as to the relationship between INR and bleeding or rethrombosis [6]. On the other hand, a lack of reliability was noticed at the onset of warfarin therapy. Also, the ISI provided by the manufacturer may be a source of error. On the other hand, a loss of accuracy was reported when thromboplastins with high INR values and/or automatic clot detectors were used [7]. Finally, it should be noted that the PT is not sensitive to the effect of heparin. This in itself shows that it is a correlate to antithrombotic action only if this is obtaiend by oral anticoagulation, but cannot be the indicator of the pathophysiological mechanism behind it, otherwise it would have to react to heparin as well.

Activated Partial Thromboplastin Time (aPTT)

Principle of the Test

The aPTT represents the lag phase before explosive thrombin generation in an intrinsically triggered plasma sample, i.e. after recalcification in the presence of sufficient procoagulant phospholipid and a trigger of contact activation. The clotting factors involved are XII, XI, X, IX, VIII, V, II and I. The rate-limiting step in this process in all probability is the feedback activation of factor VIII by thrombin. This makes it an indicator of antithrombin activity, at least in plasma in which the participating clotting factors are normally present. Factor VIII cannot participate in the clotting cascade unless it is bound to phospholipid. Plasmatic, unactivated factor VIII does not bind to phospholipid because it is bound to von Willebrand factor. Its activation therefore can only be done by thrombin in solution. The presence of heparin decreases the lifetime of thrombin in solution and hence makes it more difficult for thrombin to activate factor VIII and thus retards the thrombin burst and prolongs the lag time [8, 9]. Factor V can adsorb to phospholipid and can be activated by membrane-bound meizothrombin. These membrane-bound processes are essentially immune to heparin action. This explains why thromboplastin-induced clotting times are insensitive to heparin whereas the aPTT is.

Performance

aPTT is an aspecific test, sensitive to all disturbances of the intrinsic clotting system. As a consequence of its mechanism of action, it is particularly sensitive to variations in the concentration and/or activity of factor VIII. It shows large interindividual variation and is strongly reagent and instrument dependent [10].

Interlaboratory variability of aPTT is excessive, as revealed by national and international surveys [11]. A collaborative study was aiming to standarize aPTT monitoring of UFH by use of a reference aPTT reagent. The study concluded that each laboratory should perform its own local aPTT system calibration. The study also shows that aPTT standardization is obviously not simple, if at all possible. It is strongly felt that a reliable overall test to monitor heparin therapy is strongly needed [12], but there is considerable doubt whether the approach that was successful with the PT in oral anticoagulant will serve as useful purpose in this case.

aPTT in the Control of Heparin Therapy

Despite these drawbacks, the aPTT is universally used as an indicator of the therapeutic level of heparin-mediated anticoagulation. As a rule of thumb, doubling the aPTT is thought to represent adequate heparin administration. This concept of a defined therapeutic range is based on experimental studies in animals [13] and on a limited subgroup analysis of two prospective cohort studies in man [14, 15]. The results of these studies have led to the recommendation that the therapeutic range of heparin should be an aM of 1.5 to 2.5 times the control, which is equivalent to a heparin level (measured by protamine titration of the thrombin time) of 0.1–0.4 IU/ml or, in terms of anti-factor Xa activity, of 0.3–0.7 U/ml. For the most commercial aPTT reagents in use at present, the lower limit of the therapeutic range is over 1.5 and close to 2.0 times prolongation.

How does the aPTT perform as a monitor of heparin administration? In a recent study, significant differences in respect of heparin responsiveness were found between six aPTT reagents [16]. In addition, the limitation of aPTT for heparin monitoring was highlighted by the poor correlation between heparin concentration by protamine titration and aPTT ratio [16]. In some cases, high aPTT values are associated with subtherapeutic heparin levels [17]. This lack of association in these circumstances could be due to underlying diseases which affect the aPTT only.

It has been observed that a similar dosage of UFH produces significantly different prolongations in aPTT in different patients. The most likely explanation is the nonspecific binding of UFH to plasma proteins that neutralizes its anticoagulant effect. A number of these heparin-binding proteins are acute-phase reactants that tend to be elevated in sick patients. Another protein that neutralizes heparin is platelet factor 4, a platelet constituent that is released during platelet activation in vivo (thrombosis!) and in vitro (blood handling and storage). In patients with heparin resistance as assessed by the aPTT, the heparin level measured by the anti-Xa assay is a more relevant marker of the effectiveness of the treatment, since this test is relatively unaffected by the high levels of procoagulants that shorten the aPTT [16].

Recently, a formal review of the literature was performed on all available studies that provided information on the relationship between the risk of recurrent venous thromboembolism and the early aPTT response (within 24–48 h of initiation of treatment) to continuous intravenous heparin. More than 700 studies were identified, of which 50 were considered sufficiently informative for the overview. No convincing evidence shows that the

risk of recurrent venous thromboembolism is critically dependent on achieving a therapeutic aPTT result within 24–48 h [17].

aPTT as a General Indicator of Anticoagulant Therapy

The criterion of doubling the aPTT is sometimes, thoughtlessly, applied to other anticoagulants like direct thrombin inhibitors. This overlooks the fact that the mechanism by which such inhibitors prolong the aPTT is significantly different from that of heparin. Unlike heparin, such inhibitors do act on meizothrombin and therefore on the prothrombinase complex. This shows, even without going into details, that the dose-effect relationship must be quite different.

It is generally recognized that low-molecular-weight heparins with a high anti-factor Xa activity, and the synthetic pentasaccharides a fortiori, exert their antithrombotic action at doses that hardly prolong the aPTT. This in the first place indicates that the aPTT does not directly represent the antithrombotic effect, so that it is not allowed to assume that two times prolongation of the aPTT can be used with other anticoagulants than with UFH.

Thrombin Clotting Time (TCT)

TCT is based on the fact that a certain amount of thrombin is required over a certain time in order to clot a fibrin-containing plasma sample. The more thrombin is present, the shorter the time. Thrombin added to plasma will disappear with a velocity dependent upon the antithrombin activity, i.e. upon the presence of heparin. Clotting will be faster when thrombin persists longer, i.e. the less heparin there is. This test is very sensitive and relatively specific for the antithrombin effect of heparin. There are differences in heparin sensitivity between various forms of TCT, that can be ascribed to variations in thrombin concentrations, thrombin type (bovine or human), and addition or not of calcium [18]. Very low concentrations of UFH can be detected when a low thrombin concentration and no calcium is used. Conversely, high concentration of thrombin and addition of calcium is necessary to assess high concentrations of UFH [19].

TCT is influenced by other factors than heparin, such as fibrin degradation products. Also variations in pH or temperature influence the results [20]. The reliability of TCT in monitoring of heparin treatment has been a matter of dissension. It has been advocated by some authors, while others have underlined cautiousness and doubt [21, 22]. One point seems clear: if only one single thrombin concentration is used, only a narrow range of heparin concentrations can be accurately assessed. This range is somewhat enlarged by the addition of calcium [23].

Activated Clotting Time

The activated clotting time is a whole blood clotting time in the presence of a contact activator such as celite [24]. It is a rapid and simple test that can be carried out at the bedside and in the operating theater. Indeed its widest use, in the USA, is to monitor heparin therapy during extracorporeal circulation and coronary angioplasty [25]. The test is essentially an aPTT carried out on whole blood. Like the aPTT it covers an acceptable range of heparin levels. It carries the same drawbacks as the aPTT and is also strongly influenced by the conditions of blood sampling [26].

Ecarin Clotting Time

Ecarin is a snake venom enzyme derived from the viper *Echis carinatus*, which converts prothrombin into meizothrombin and other intermediates. The thrombin intermediates exhibit thrombin-like activity on the fibrinogen in the sample. While direct thrombin inhibitors are able to inhibit these thrombin intermediates, antithrombin has no effect and consequently heparin not either. Recently, ecarin clotting time has been proposed for the specific measurement of the effect of hirudin and related direct antithrombin agents [27]. This method is reported to show linear correlation with the anti-IIa activity of hirudin, to be highly reproducible, simple to perform and reliable for bedside monitoring of hirudin [28, 29]. Critical reviews of its performance in practice are still lacking.

Heparin Anti-IIa and Anti-Xa Activities

The specific action of heparin is that it accelerates the inactivation of thrombin and factor Xa in plasma by antithrombin. This property can be used to estimate these specific activities of heparin in a sample. These methods are based on inhibition of a known amount of factor Xa or thrombin, and are usually based on measuring residual factor Xa (thrombin) activity after a fixed incubation time, using a photometric determination with a synthetic peptide substrate to determine residual activities. The use of a photometer reduces the observer bias. These tests are as sensitive as and more specific than the aPTT [30]. Assay of anti-Xa activity is the most widely used method for assessing low-molecular-weight heparin ex vivo. The results are expressed in anti-Xa international units per milliliter of plasma. Here an important artifact must be noted. In plasma, in the presence of Ca^{2+} ions, the anti-factor Xa activity of UFH, and hence of the international heparin standard, is twice higher than in an anti-Xa test situation where Ca^{2+} is absent. This is not the case, however, for low-molecular-weight heparins. Hence, the latter are measured against a 'handicapped' standard and overestimated. The effect causes 1.5–2.0 times too high anti-factor Xa values [31]. It should be noted that the use of the international standard for low-molecular-weight heparins does not abolish this error because the activity of that standard itself has been determined against the UFH standard. Amidolytic methods may differ as to the reagents used. The addition of purified antithrombin to the test plasma is recommended by some authors and rejected by others [32]. A well-designed test renders, however, always the catalytic activity of the heparin present in the sample, independent of other components that may be present.

Endogenous Thrombin Potential (ETP)

The thrombin-generating capacity of plasma is one of the main determinants of hemostasis and thrombosis [33]. Clotting times measure the onset of thrombin generation, the moment that about 1 unit of thrombin (~ 10 n*M*) is formed. Most thrombin, up to 100–250 n*M*, appears after the clot is formed. In vivo this thrombin will still act on its numerous substrates and exert a prothrombotic action. It has been shown that the enzymatic potency of generated thrombin is best expressed as the area under the thrombin generation curve, or ETP. The ETP can be determined from a classical thrombin generation curve, but this is unsuitable for routine purposes. A simplified and automated test has been described [34]. This parameter might possibly be representing the essential variable

decreasing in drug-induced hypocoagulability and increasing in hypercoagulability [34].

Preliminary results were obtained with this ETP assay as a routine procedure. The ETP appears to be decreased to between 20 and 40% of normal by oral anticoagulation in the therapeutic range (INR = 1.5–3.0) and by heparin administration in the therapeutic range (aPTT = 1.5–2.5 control). It also indicates the hypocoagulability obtained in mixed treatment, common in the 1st week of treatment of venous thrombosis, and in which none of the currently used tests gives reliable guidelines. The ETP is increased in untreated subjects with congenital antithrombin deficiency and in women using contraceptives. It is also increased in patients with deep vein thrombosis and in coronary infarction after the acute phase [28]. Extensive large-scale clinical trials will still be necessary to demonstrate the feasibility and the ability of the ETP as a general indicator of anticoagulation or hypercoagulable states.

Conclusion

Laboratory monitoring of anticoagulant therapy has its roots in the past, and is more often than not founded on tradition rather than on well-established data. Secondary rigorous standardization may (as in the case of PT) or may not (as in the case of aPTT) ameliorate the situation. In any case, the need of an adequate assay to monitor anticoagulant/antithrombotic therapy is obvious. This test should be easy and cheap and should represent an essential parameter of the coagulation system and therefore be applicable to all anticoagulants in the same way. No such test is available at this moment, but the ETP proposes as a candidate worthy of clinical testing.

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